MOLECULAR GENETIC POPULATION STRUCTURE IN STEELHEAD/RAINBOW TROUT FROM THE SANTA YNEZ RIVER 1994- 1997

Appendix F

Prepared for:

SANTA YNEZ RIVER TECHNICAL ADVISORY COMMITTEE

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Molecular Genetic Population Structure in Steelhead/Rainbow Trout (Oncorhynchus mykiss) from the Santa Ynez River, 1994-1997

by

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INTRODUCTION

A total of 95 *Oncorhynchus mykiss* fin clips taken from fish collected in the Santa Ynez River, 1994-1997, were analyzed for molecular genetic population structure in my laboratory at Hopkins Marine Station for the Santa Ynez River Technical Advisory Committee (SYRTAC). For this study we amplified D-loop nucleotide sequence (188 base pairs) and ten nuclear microsatellite loci from DNA extracted from each fin sample. Previously published/reported genetic data for Santa Ynez steelhead/rainbow trout are summarized in Appendix III.

These genetic markers represent two different molecular systems found in the salmonid genome with potentially different selection mechanisms reflected in their genetic diversity. Mitochondrial DNA (mtDNA) is a maternally inherited, extra-nuclear locus which has been used extensively for studies of conservation genetics and genetic diversity in vertebrates since the early 1980's. The D-loop sequence used in this study has been well documented in the published literature as one of the most diverse regions of DNA sequence available from teleost fishes (including salmon and trout) due to its relatively fast mutation rate (Lee et al. 1995; Nielsen et al. 1998). The term "relatively" should be taken at the correct scale, however. Most mtDNA divergence leading to unique haplotypes as described in this report is thought to have occurred during the mid- to late-Pleistocene, or 70,000 to 250,000 years ago (Avise 1994).

Pleistocene glaciation had unprecedented impacts on the ecology and genetic structure of North American vertebrate species (Pielou 1991). Fish species suffered long term disruptions due to glacial cover of freshwater habitats, formation and failure of ice dams, drainage shifts, and sudden emptying or flooding of ice-margin lakes. Much of the current species diversity is thought to have evolved from glacial refugia found at the edge of ice sheets or in areas protected from the glacial advance (Pielou 1991; Nielsen in press). Species from glaciated regions have been shown to have reduced levels of intraspecific divergence and genetic diversity (Bernatchez et al. 1989). Recolonization from diverse refugia has led to a complex zoogeographic history for many fish species, including salmon and trout. Recent developments in genetic technology allowing thorough investigations of mtDNA lineages have given us a better understanding of the number and location of glacial refugia in wild populations of fish and their colonization trends through modern times. A strong biogeographic cline in-mtDNA haplotypes has been shown for coastal steelhead in California (Figure 1; Nielsen et al. 1994a & b, 1997a &b, 1998).

Microsatellites are short, tandemly repeated units of DNA that have been shown to be highly polymorphic in plants and animals. Fast mutation rates leading to high

levels of variation and a broad genomic distribution have made microsatellites important genetic markers for studies of parentage, genetic linkage, and population structure in many organisms (Jarne and Lagoda 1996). Mutation rates in microsatellites have been shown to be on an order of magnitude faster than most mtDNA markers making them important in studies of evolution that has occurred since the Pleistocene. Recent estimates of divergence times for microsatellites in humans by Goldstein et al. 1995b, place allelic changes on the scale of tens-of-thousands of years, a period covering most of the recent tectonic uplifting activity along the coast of California. This level of divergence makes these markers appropriate for question of genetic diversity involving recent anthropomorphic manipulations of fish populations such as hatchery propagation or habitat alteration due to dams and urbanization of river channels (see Nielsen 1996; Nielsen et al. 1997a & b).

Molecular genetic comparisons using these two different molecular systems were made among sample populations and other reference populations of California steelhead/rainbow trout analyzed for the same markers in the past in my laboratory. I used comparisons of allelic and haplotype frequency data, genetic distance measures, and analyses of population independence to compare genetic markers among subgroups from the SYRTAC samples and between the SYRTAC samples and other California *O. mykiss* populations.

MATERIAL and METHODS

Sample Collections

Ninety-five *O. mykiss* fin clips collected by SYRTAC were sent to our laboratory in 1997. These fish included samples collected 1994-1997 from Alisal Creek (N=17); Hilton Creek (N=36); Long-pool/spill basin (N=10); Salsipuedes Creek (N=31); and San Miguelito Creek (N=1; Table 1).

Fish collected from Alisal Creek, San Miguelito Creek, Devils Creek, and the Whale Rock Hatchery were collected above passage barriers. Comparison collections available in our laboratory for the same molecular markers included in analyses of population independence and genetic distance analyses were *O. mykiss* samples collected from Hilton Creek in 1995 (N=11) by the California Department of Fish and Game (CDFG); samples taken by Giles Manwaring from southern steelhead in Malibu Creek in 1992-93 (N=13); rainbow trout samples collected by the USFS in Devil's Creek

Figure 1. Map showing biogeographic cline in mtDNA haplotypes along California's Pacific coast (from Nielsen in press).

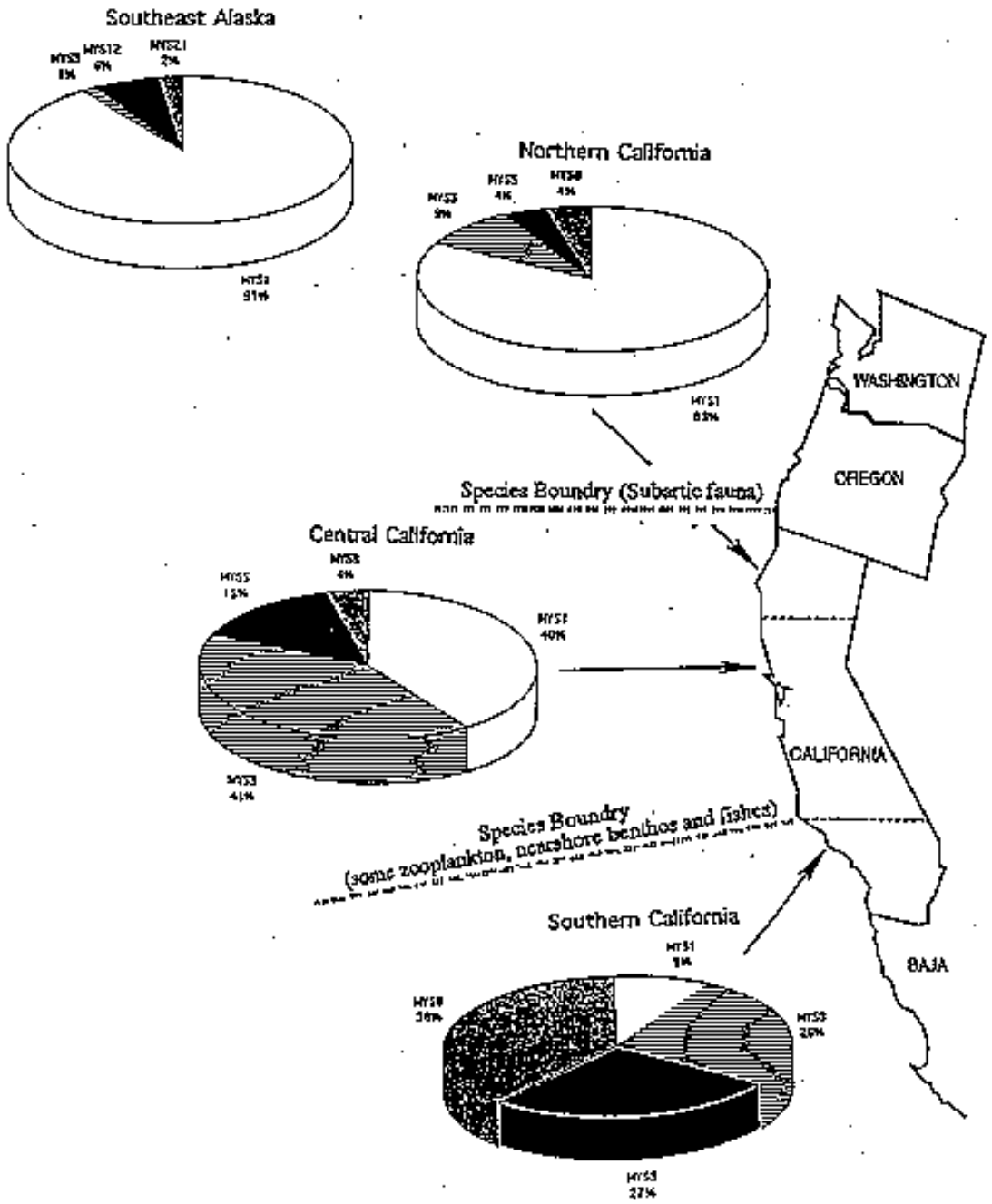


Table 1. General sample collections and the number of steelhead/rainbow trout used in these analyses.

Population	Sample year (s)	Sample count (N)	Above passage barrier
SYRTAC			
Alisal Creek	1995	17	Yes
Hilton Creek	1994	1	No
	1995	24	No
	1997	11	No
Long pool/spill basin	1997	10	No
Salsipuedes Creek	1995	3	No
	1996	3	No
	1997	25	No
San Miguelito Creek	1997	1	Yes
subtotal (SYRTAC)		95	
Reference collections			
Hilton Creek (CDFG)	1995	11	No
Devils Creek (USFS)	1995	7	Yes
Malibu Creek	1992-93	13	No
Whale Rock Reservior	1992	33	Yes
North coast streams	1992-93	27	No
subtotal (reference)		91	

from the upper Santa Ynez watershed in 1995 (N=7); putative “landlocked” steelhead from Whale Rock Reservoir collected in 1992 (N=33); and steelhead/rainbow trout fin clips collected from nine northern California coastal drainages, 1992-93, (Albion River, Cottoneva Creek, Garcia River, Gualala River, Howard Creek, Middle Fork Eel River, Navarro River, Usal Creek, and the Van Duzen River; N=27). For the purposes of these analyses we pooled all of the north coast samples into one population and used this as the outgroup for our genetic distance analyses of the SYRTAC samples.

North coast steelhead microsatellite data given in Appendix I have been previously published in part by JLN (Nielsen et al. 1997a & b). Other raw genetic data from the reference collections used in this report remain the property of the collecting agency and are not included here. These data may be available upon request from the collecting agency. The reference collections are offered here as comparisons made among sample populations taken recently in the same general geographic area as the SYRTAC samples. They are especially useful for microsatellite analyses where limited data on California's *O. mykiss* have been published to date (Nielsen et al. 1997a & b).

Mitochondrial DNA

Total genomic DNA was extracted from *O. mykiss* fin clips using Chelex-100 (BioRad) and/or cesium chloride purifications (Nielsen et al. 1998; Carr and Griffith 1987). Amplification of mitochondrial DNA (mtDNA) control-region sequence according to methods given in Nielsen et al. (1994a) were successful in all fin clips from the SYRTAC collection. Primers used in this study (P2 and S-phe) are known to allow the amplification of a highly variable segment of mtDNA control region in salmonids (Nielsen et al 1994a & b; Nielsen et al. 1997; Nielsen et al. 1998). This segment of mtDNA contains 188 base pairs (bp) of the O. mykiss control region and 5 bp of the adjacent phenylalanine tRNA gene. Primer sequences, amplification and sequencing protocols, and the complete sequence amplified in this region in O. mykiss are given in Nielsen et al. 1994a.

Nomenclature for mtDNA control region haplotypes follow those given in Nielsen et al. 1997a. I used an unbiased estimate of the Fisher's exact test based on a Markov chain adaptation of row-by-column contingency tables (GENEPOP V2.0; Raymond and Rousset 1995a) to test for independence in mtDNA haplotype frequencies found among steelhead/rainbow trout populations used in this study. This test provides the probability of being wrong when Ho (i.e. rows and columns are independent) is rejected (Raymond and Rousset 1995b). Haplotype frequency analysis was done using ARLEQUIN 1.0 (Schneider et al. 1997 http://anthropologie.unige.ch/arlequin) and a genetic distance

tree for linearized Fst values among sample populations (SYRTAC and reference data) was calculated using PHYLIP (Felsenstein 1993).

Microsatellite Loci

Ten microsatellite loci developed by other research laboratories were chosen for these analyses based on their high level of polymorphism in previous studies of steelhead/rainbow trout done in our laboratory. The Omy-series of microsatellites was developed specifically for *O. mykiss*; the Oneμ-series was developed for sockeye salmon (*O. nerka*); Ots-series microsatellites were developed for chinook salmon (*O. tshawytscha*); and the Ssa-series was developed for Atlantic salmon (*Salmo salar*). Amplification of microsatellite loci follow methods given in Nielsen et al. 1997a, except that each 7.15 μl PCR reaction contained 67 μM Tris-HCL (pH 8.8), 6.7 μM $MgCl_2$, 16.6 μM $(NH_4)_2SO_4$, 10 μM β-mercaptoethanol, 1 μM each of dGTP, dATP, dTTP, and dCTP, 1 μM of each primer, 0.15 units of Taq polymerase, and μl of Chelex-100 extracted DNA.

For each locus polymerase chain reaction (PCR) conditions and the color of the fluorescently labeled reverse primer are listed in Table 2. Microsatellite alleles were run on a 6% polyacrylamide gel. Prior to loading the gel, 1μl PCR product was added to 4 μl of loading buffer

Table 2. Polymerase chain reaction (PCR) conditions used to amplify 10 microsatellite loci in Santa Ynez River steelhead/rainbow trout. Primer concentrations were 1μM for all reactions. Loci are listed by fluorescent labeled reverse primer.

*Tan °C/cycle	6Fam-blue	Tet-green	Hex-yellow
52°/30	Oneμ14 Omy27	Ots1	Oneμ11 Ssa289
52°/32	Omy77 Oneμ2	Ssa14	Omy325 Oneμ8

*Tan = annealing temperature

containing 1 µl 50 mg/ml Blue Dextran, 2.5 µI diformamide, and 0.5 µl ABI Genescan 500 (Applied Biosystems). All microsatellite gels were run on an ABI 373 automatic sequencer adapted for microsatellite analysis.

Microsatellite gels were read using ABI Prism's GENOTYPER software (1996). Microsatellite loci were run individually in separate PCR reactions to determine the maximum allelic size distributions found in Santa Ynez steelhead/rainbow trout. Allele sizes for each locus were established following an analysis of variance in allele size estimates derived from GENOTYPER. The size reported here for each microsatellite allele was equal to the size of the total product amplified (including amplified primer sequence). Known *O. mykiss* samples and commercial size standards were rerun on each gel for size standardizations among gels.

Tests for population independence using microsatellite allelic frequencies were performed using GENEPOP. Fisher's exact tests were run on all possible pairs of fish populations for each locus and for all loci combined. Statistical significance levels (initial $\alpha = 0.05$) were set using sequential Bonferroni tests (Rice 1989). Pairwise genetic distance matrices were calculated using the measure $\delta\mu^2$ (delta mu squared; Goldstein et al. 1995a), using MICROSAT V 1.4 available from Dr. E. Minch, Department of Genetics, Stanford University (http://lotka.stanford.edu/distance.html).

This distance measure assumes a linear expectation of the average squared distance for each locus (assuming no correlation between mutation rate and repeat score) and uses the arithmetic average of mutation rates across loci. This statistic is equivalent to a general analysis of variance using the sum of squares of differences in allelic size within each locus for each population, and the average squared difference between all possible pairs of populations. These estimates are used to obtain an estimate of variance in allele size in the total population. Goldstein's distance measure maintains an estimate of mutation rates under an expectation of a strict, single-step ($\pm$ one repeat unit) shift for each mutation event. Fst and mean heterozygosity for the 10 microsatellite loci were calculated using MICROSAT with expected equilibrium values developed for the stepwise mutation process.

Distance data were used to generate an unrooted consensus neighbor-joining tree using NEIGHBOR81 and CONSENSE applications from PHYLIP (Felsenstein 1993) comparing the SYRTAC collection with our reference populations. One thousand replicate microsatellite distance trees were generated to obtain bootstrap estimates based on locus removal with replacement in the MICROSAT program. Bootstrap values given as percentiles were used to assess reproducibility of branching patterns found in the consensus genetic distance tree.

RESULTS

Mitochondrial DNA

Six mtDNA haplotypes were found in the Santa Ynez River samples sent to my laboratory by SYRTAC (Table 3). Haplotype frequency distributions varied among the subsample populations in this collection (Table 4). Fisher's exact tests indicated significant independence for mtDNA haplotype frequency distributions between all paired comparisons made among the SYRTAC Santa Ynez River populations (excluding the San Miguelito Creek sample where N=1), with the notable exception of the haplotype frequencies found in Hilton Creek and the adjacent long pool/spill basin (Fisher's $p = 0.16$). In year-to-year comparisons significant differences in haplotype frequencies were found between SYRTAC's Hilton Creek samples collected in 1995 and 1997 (Fisher's $p = 0.0025$).

In comparisons with available reference mtDNA collections (Appendix III; populations where $N<3$ were excluded) a lack of significant independence (Fisher's $p > 0.05$) was found in comparisons of Salsipuedes Creek and with Devils Creek ($p = 0.62$). SYRTAC Hilton Creek samples (all years combined) and CDFG Hilton Creek samples (all years combined) lacked significant independence for mtDNA haplotype frequencies ($p = 0.06$). This trend in mtDNA frequency continuity for independent collections of Hilton Creek trout held for year-to-year comparisons as well where Fisher's $p = 0.36$ (CDFG and SYRTAC 1995); $p = 0.15$ (CDFG and SYRTAC 1997).

No significant differences in mtDNA haplotype frequencies were found between Hilton Creek samples and those collected in Lake Cachuma (SYRTAC samples $p = 0.20$; CDFG samples $p = 0.11$). Mitochondrial DNA haplotype frequencies in Devils Creek fish were not significantly different from those found in the SYRTAC Hilton Creek samples ($p = 0.19$). The long pool/spill basin samples lacked mtDNA frequency independence from Lake Cachuma ($p = 0.06$) and Devils Creek ($p = 0.23$). Lake Cachuma trout lacked mtDNA independence in comparison with Devils Creek trout ($p = 0.23$). Jameson Reservoir fish and the collection made in Franklin Creek lacked significant mtDNA frequency differences in comparison with adult fish collected in the Santa Ynez River (1993-94; Jameson Reservoir $p = 0.36$; Franklin Creek $p= 0.08$).

Table 3. Mitochondrial control region variable sites and nucleotide changes (bold) found in relation to MYS1 in the upper Santa Ynez River steelhead/rainbow trout 1994-1997.

mtDNA type	base pair no. 1021	1086	1103	1106	1109	1147
						Digby et al.'92
MYS1	T	**T**	A	A	G	G
MYS3	T	**T**	A	A	A	G
MYS5	T	C	G	C	G	A
MYS8	T	C	A	C	G	A
MYS12	T	C	A	C	G	G
MYS14	C	T	A	A	A	G

Table 4. Mitochondrial haplotype frequencies found in Santa Ynez River steelhead/trout samples, 1994-1997.

Population	Year	mtDNA type 1	3	5	8	12	14
Alisal Creek	1995		17				
total		0	17	0	0	0	0
Hilton Creek	1994				1		
	1995	9	13		1		1
	1997	2	2		5	2	
total		11	15	0	7	2	1
Long pool/spill basin	1997	1	5		2	2	
total		1	5	0	2	2	0
Salsipuedes Creek	1995	1	2				
	1996			1	2		
	1997		3	4	18		
total		1	5	5	20	0	0
San Miguelito Creek	1997		1				
total		0	1	0	0	0	0
overall total		13	43	5	29	4	1

Estimates of Nm (used as a surrogate for recent gene flow among populations) calculated from haplotype frequencies by ARLEQUIN were very high in comparisons of SYRTAC Hilton Creek with CDFG Hilton Creek (Nm = 99), long pool (Nm was infinite), Cachuma Reservoir (Nm was infinite), and the 1993-94 mainstem collection by SYRTAC in the Santa Ynez mainstem (Nm was infinite). High gene flow estimates occurred between: Cachuma Reservoir and CDFG's Hilton Creek sample Nm = 23.6; Cachuma Reservoir and the Santa Ynez 1993-94 mainstem collection (Nm = 64.1; Jameson Reservoir and Alder Creek Nm = 39.45; and Fox Creek and Alder Creek Nm = 14.51. All other estimates of geneflow were less than Nm = 10, the maximum threshold suggested as appropriate for estimating connectivity in populations from geographically proximate subpopulation within a basin (Mills and Allendorf 1996).

Genetic distance analyses based on haplotype Fst values calculated by sample population for all mtDNA reference collections and SYRTAC sample locations in the Santa Ynez River (populations with 2 or less individual samples were not included) ranged from Fst = 0 (comparisons made among the long pool, Cachuma Reservoir and both Hilton Creek samples) to Fst = 7.8 (El Jaro/Salsipuedes and Alisal Creek). A mtDNA consensus neighbor-joining tree (PHYLIP) derived from linearized Fst values calculated by ARLEQUIN is given in Figure 2.

Microsatellite Loci

The 10 microsatellite loci used to test population structure in the Santa Ynez River trout were highly polymorphic (Table 5). The number of alleles ranged from 6 (Oneμ11) to 33 (Oneμ2), with an average of 15 alleles per locus in the Santa Ynez samples collected by SYRTAC (see Appendix I for allelic distributions found in SYRTAC samples compared to northern CA coastal collection. Allelic sizes ranged from 87 bp (Omy325) to 308 bp (Oneμ2). Mean Fst for the 10 loci combined was 0.11 (range: 0.03 (Omy27) to 0.21 (Oneμ8)). Average heterozygosity for the 10 loci was 0.62 (range: 0.45 (Omy27) to 0.80 (Oneμ2)).

Fisher's exact tests of population independence were performed on paired comparisons among the SYRTAC samples and the northern California reference collection using 10 microsatellite loci (Table 6). One fin clip collected by SYRTAC in Hilton Creek, 1994, represented the only fish from the SYRTAC collection that showed significant lack of independence for all 10 loci in comparisons with north coast steelhead (mean Fisher's p = 0.44; see Table 6). Year-class variation for the 10 microsatellite loci

Figure 2. Consensus unrooted neighbor-joining tree (PHYLIP) derived from genetic distance estimates based on pairwise Fst values for mtDNA haplotype frequencies using ARLEQUIN in trout populations from the Santa Ynez River. The number of samples (n) follows each site location.

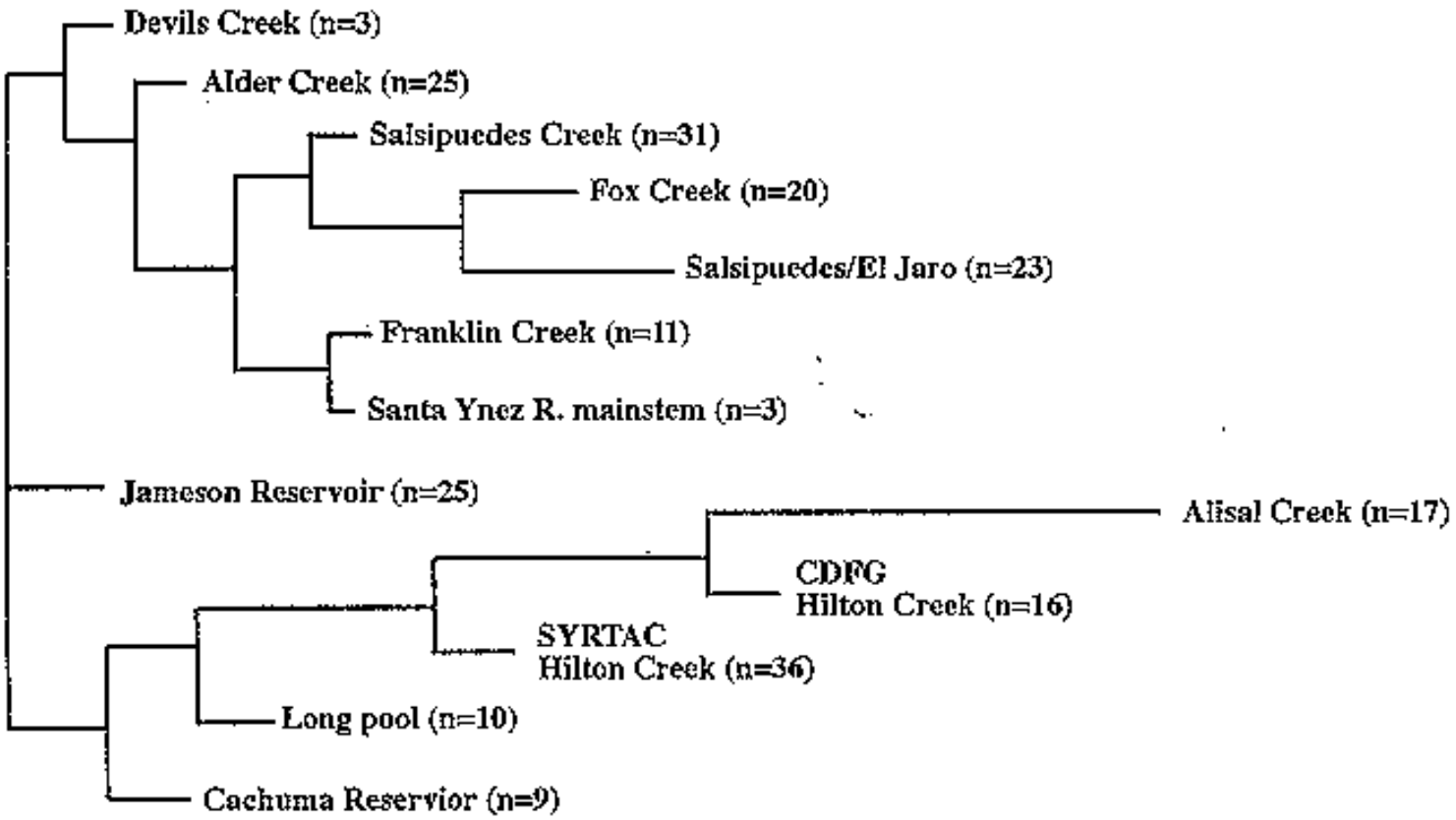


Table 5. List of 10 microsatellite loci and their source publications amplified from Santa Ynez steelhead/rainbow trout, 1994-1997, and north coast steelhead populations. Size S.D. represents the mean standard deviation calculated for allelic size estimates made at each allele for each locus amplified from all steelhead/rainbow trout samples used in this study.

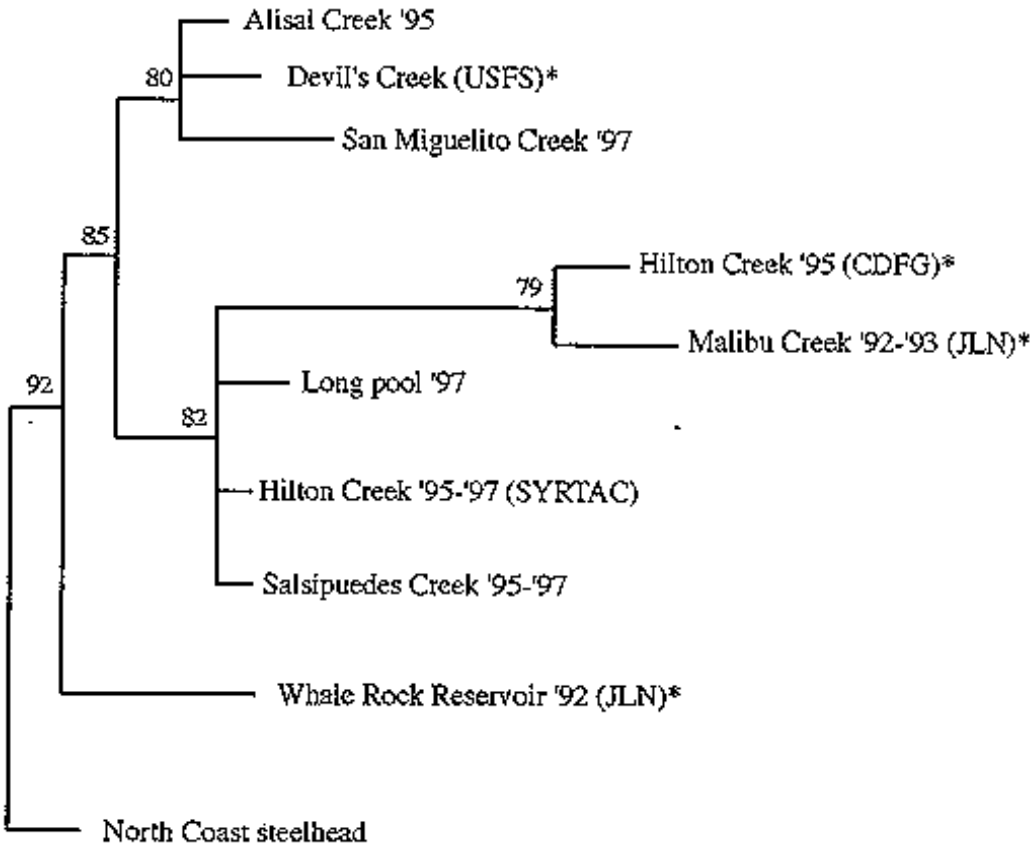
Locus	Source	Number Alleles	Allelic Size (bp)	Size S.D.(bp)
Omy27	M. O'Connell pers. comm.	10	97-115	0.24
Omy77	Morris et al. 1996	28	93-153	0.26
Omy325	M. O'Connell pers. comm.	22	87-145	0.36
Oneμ2	Schribner et al. 1996	34	204-308	0.22
Oneμ8	Schribner et al. 1996	16	146-190	0.36
Oneμ11	Schribner et al. 1996	6	141-153	0.17
Oneμ14	Schribner et al. 1996	9	145-171	0.20
Ots1	M. Banks pers. comm.**	17	151-243	0.24
Ssa14	McConnell et al. 1995	14	126-166	0.21
Ssa289	McConnell et al. 1995	8	108-124	0.26

*M. O'Connell, Guelph University, Ontario Canada
**M. Banks, University of California, Davis

Table 6. Genetic distance values ($\partial\mu 2$) calculated using Goldstein et al. (1995) on 10 microsatellite loci in paired comparisons of the SYRTAC steelhead/rainbow trout samples and north coastal California steelhead samples are given below the diagonal. Above the diagonal are the numbers of microsatellite loci showing significant ($p<0.025$) independence between paired comparisons based on GENEPOP's Fisher's exact tests.

	Population									
Population	1	2	3	4	5	6	7	8	9	10
1) Alisal Creek '95	-	1	10	8	2	9	6	5	9	10
2) Hilton Creek '94	13.38	-	0	1	0	0	0	0	1	0
3) Hilton Creek '95	4.14	14.00	-	2	0	1	0	0	9	9
4) Hilton Creek '97	7.28	4.75	4.83	-	1	1	2	5	8	7
5) San Miguelito Creek '97	4.26	16.03	8.19	13.22	-	0	0	0	1	1
6) Long pool '97	5.40	9.00	2.38	1.15	11.19	-	0	2	9	8
7) Salsipuedes Creek '95	18.23	14.71	11.31	5.81	30.41	7.95	-	0	2	4
8) Salsipuedes Creek '96	18.97	19.58	15.84	17.15	18.48	14.13	37.70	-	0	6
9) Salsipuedes Creek '97	3.62	7.19	5.68	3.92	7.39	3.39	17.80	9.08	-	8
10) North coast streams '92-93	3.40	8.85	3.11	6.03	3.80	5.34	17.40	14.56	4.44	-

Figure 3. Consensus neighbor-joining tree derived from genetic distances ($\partial\mu 2$) for 10 steelhead/rainbow trout populations surveyed at 10 microsatellite loci (see text). Bootstrap values (% of 1,000 trees) less than 75% were collapsed due to small sample sizes in many of these populations.



* Data used to analyze these populations remain discretionary and the property of the collecting agencies.

amplified from fins collected in 1995 and 1997 in Hilton Creek was not significant (Fisher's combined p = 0.15). Large differences in sample size prevent legitimate statistical year-class comparisons among the other SYRTAC fish populations.

Delta mu ($\delta\mu^2$) genetic distance analyses among the SYRTAC trout populations ranged from $\delta\mu^2$ = 37.70 (Salsipuedes Creek 1995 and 1996 samples) to $\delta\mu^2$ = 1.15 (long pool/spill basin 1997 and SYRTAC's Hilton 1997 samples; Table 6). Neighbor-joining analysis of the $\delta\mu^2$ distance measures including all of the reference collections, demonstrated two genetic groupings with separation supported by 85% of the bootstrap trees (Figure 3). Alisal Creek, San Miguelito Creek, and Devils Creek (USFS) made up one group, while both Hilton Creek samples (SYRTAC combined year-classes and CDFG), Malibu Creek, long pool, and Salsipuedes Creek (SYRTAC combined year-classes) made up the other.

DISCUSSION

Comparisons of SYRTAC sample populations by site locality and year showed the important influence sample size can have on these types of analyses. Most statistical theory and data simulation studies suggests 40-60 individuals/population for best results when analyzing population structure with microsatellite loci (see Takezaki and Nei 1996 and literature therein). The largest Fst and $\delta\mu^2$ distance values were calculated in comparisons where at least one population contained only a few individual suggesting significant sample-size effects. Combining samples across years for individual tributary or stream populations gave better results in our neighbor-joining analyses.

The controversy over mitochondrial vs. nuclear (i.e. microsatellite) DNA analyses continues in the genetics community. The evolutionary mechanisms in repeat DNA remain unknown and, therefore, the assumptions built into their analyses are controversial. I have published significantly using both methods given here (see literature cited). Results documenting population genetic structure within the Santa Ynez River basin were not congruent for these two markers. This could result from several conditions or constraints on the data. In this study both methods were applied to different population sets since most of the mtDNA reference populations have not been analyzed for microsatellite diversity at 10 loci (see Figures 2 and 3). It is difficult to support variation in genetic structure based on differences in mutation rates between the two markers or sexually dimorphic gene-flow (i.e. more straying of males within the basin). As mentioned above sample size is a problem at many of the locations used for this study. Errors resulting from low sample number will, however, tend to have more effect in microsatellite analyses than in mtDNA sequence data due to their variable

mutation rates. I anticipate increased sample sizes (at least 40 fish per sample location per year) would bring congruence between these two genetic markers in their depiction of within basin population genetic structure.

Two issues concerning the microsatellite analyses were important enough for me to give them computational consideration. First, recent studies of microsatellite loci have shown null alleles (Omy77 and Oneμ14) and size homoplasy (Oneμ11) in bottlenecked populations of *O. mykiss* in Alaska (JLN and W. Ardren, unpublished data). I ran $\delta\mu^2$ genetic distance analyses on the SYRTAC samples without each of these loci and without all three loci combined to analyze the relative contribution of each locus on the overall findings. These analyses did not change the architecture of the resulting genetic distance tree or the relative relationships found among the Santa Ynez River samples. Variation found at each locus acted on all populations with equal effect. Similar results for these loci in other studies on going in my laboratory show similar effects (Nielsen in press; Nielsen et al. submitted). Tree branch lengths did change, however, due to the shifts in analysis of variance contributed by each locus. These changes would typically affect an interpretation of deep evolutionary nodes, but the Santa Ynez River populations are so closely related that branch lengths~were~not considered significant in either case (with or without the questioned loci).

I used a second method of analysis of genetic distance for microsatellite data (Nei's chord distance) that is based on the infinite allele model of evolution as opposed to $\delta\mu^2$'s single-step model. Nei's measure ranged from 0 - 1.17 in the Santa Ynez samples, but was generally directly correlated to the $\delta\mu^2$ values given here, suggesting that the mutation model is not as important in recently diverged populations as in analyses involving more distantly diverged populations (see Takazaki and Nei 1996). Nei's mean Fst for these 10 microsatellite loci was 0.12, very similar to the value calculated by $\delta\mu^2$ (Fst = 0.11).

It was interesting that I was unable to differentiate the one fish caught in Hilton Creek (1994) that carried mtDNA haplotype MYS8 (most commonly found in southern California steelhead) from north coast steelhead for any of the 10 microsatellite loci. This shows the error that can easily be made using genetic analyses without consideration of the sampling properties inherent in the system of markers used to define subgroups of fish as independent populations (see Cummings et al. 1995). While mtDNA haplotype MYS8 dominated the Whale Rock Reservoir population collected in 1992, these fish clearly had a mixed ancestry when we looked at the nuclear genome (Nielsen et al. 1 997b). These examples show the importance of

looking at sufficient sample sizes for both mtDNA and nuclear markers when examining genetic population substructure within a basin.

Due to a natural genetic heritage primarily derived from Sacramento River rainbow trout, hatchery trout in California are dominated by two haplotypes MYS1 and MYS3. It is important to note, however, that haplotypes MYS1 and MYS3 do not necessarily indicate hatchery-derived fish in southern California streams. Despite the fact that their frequency of occurrence declines in southern streams, these haplotypes have been found throughout the species range as far south as Baja California (Nielsen 1998). A wild-caught fish cannot be determined to be hatchery derived simply by exanination of their mtDNA haplotype. The probability of hatchery origins increases in fish carrying MYS1 or MYS3 haplotypes, but wild origins cannot be ruled out in these lineages, even in southern California. My laboratory is working on a series of microsatellite loci that seem to contain diagnostic alleles for the Mount Shasta, Hot Creek, and Whitney Hatchery rainbow trout strains. Completion of this work (expected in early 1999) will provide tools for hatchery vs. wild comparisons within California coastal rainbow trout populations and allow estimates of the level of introgression by hatchery fish among stocks subjected to supplementation over time.

Genetic distances calculated between the 1995 (N=3) and both the 1996 (N=3) and 1997 (N=25) samples collected in Salsipuedes Creek were quite high ($\delta\mu^2$ = 37.7 and 17.5 respectively). Despite small sample sizes for 1995 and 1996, this seems to indicate year-class structure or sampling problems in this tributary. Year-class structure and/or sampling problems were also found in SYRTAC's 1995 (N=24) and 1997 (N=11) Hilton Creek collections. For all year-classes combined we found no significant differences between the SYRTAC Hilton Creek collections and those sent to my laboratory by CDFG with both Hilton Creek collections occurring on the same branch in Fst distance analysis, only 64% bootstrap support for separation in the microsatellite neighbor-joining tree, and high Fisher's combined tests p-values among the various Hilton Creek collections.

Fst distance analyses of haplotype frequencies showed upper and lower basin substructure for mtDNA with the notable exception of Salsipuedes Creek which claded with the upper basin fish populations (Figure 2). Two well supported genetic clades based on nuclear microsatellite allelic structure shown in the lower Santa Ynez River trout samples gave support for genetic associations among Malibu Creek steelhead and trout from Hilton Creek, the long pool, and Salsipuedes Creek. Alisal Creek, San Miguelito Creek, and Devil's Creek trout were significantly different in microsatellite allelic structure from known anadromous steelhead populations in Malibu Creek. No

Santa Ynez River reservoir fish were included in these microsatellite analyses, but a previous study of Cachuma and Jameson Reservoir samples for three microsatellite loci showed closer genetic affinity between reservoir fish and trout from habitats currently closed to ocean access due to dams (Nielsen et al. 1997b).

The difference in genetic substructure found for the two molecular markers could be due to variation in life histories (i.e. time since anadromony) above and below dams, or to hatchery introgression sometime in the recent past that has affected some habitats more others. Hatchery introgression may have resulted in significant males genetic contribution in reservoirs and downstream tributaries (as represented by microsatellite data), with limited female gene flow leading to the preservation of population substructure in the Santa Ynez River based on mtDNA analyses. It is also possible that two distinct lineages (i.e. independent steelhead and rainbow trout populations) co-occur naturally within the basin. The lack of "diagnostic" alleles fixed for either of these two life histories, however, argues against this last hypothesis.

Sample sizes analyzed for genetics were small for many of these populations and prevent my making any further speculation on the cause of population differentiation using either marker. I would suggest that a broader overview of the population genetic structure for *O. mykiss* in the Santa Ynez River would be very helpful in resolving the effects of past hatchery supplementation, the development of supplemental broodstocks for enhancement, and in dosing of an appropriate conservation plan for this basin. We especially need additional genetic data and samples from the upper headwaters of this basin to determine if relic gene-pools found in resident fish in the waters can provide material for supplementation of anadromous stocks in the Santa Ynez River. A follow up study with sample sizes on the order of 40-60 fish per putative population or sample site (i.e. tributary or mainstem locations) would give sufficient statistical rigor to address this issue using microsatellites. Such a study should be done cooperatively between the diverse agencies involved in the recovery of southern steelhead in this area.

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APPENDIX I - Microsatellite allelic frequencies for SYRTAC samples and north coast steelhead.

Locus = Omy27

	97	99	101	103	105	107	109	111	113	115	Total
Alisal95	0	0	0	0	6	28	0	0	0	0	34
Hilton94	0	0	0	0	0	2	0	0	0	0	2
Hilton95	0	6	0	0	15	19	2	4	1	1	48
Hilton97	0	0	7	0	0	13	2	0	0	0	22
Miguel	0	0	0	0	0	2	0	0	0	0	2
Pool97	0	0	4	0	2	12	1	0	0	1	20
Salsi95	0	0	0	0	3	3	0	0	0	0	6
Salsi96	0	0	0	0	3	3	0	0	0	0	6
Salsi97	2	0	0	0	9	37	1	1	0	0	50
NCAsthd	0	0	5	3	9	23	14	0	0	0	54
Total	2	6	16	3	47	142	20	5	1	2	244

Locus = Omy77

	93	95	97	99	101	103	105	107	109	111	113	115	117	121	125	127	129	131	133	135	137	139	141	143	147	149	151	153	Total
Alisal95	0	0	10	4	0	2	7	1	2	5	0	0	0	0	0	0	0	0	1	0	2	0	0	0	0	0	0	0	34
Hilton94	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2
Hilton95	0	0	3	8	11	0	1	1	2	2	0	1	0	1	0	2	8	2	2	0	0	2	2	0	0	0	0	0	48
Hilton97	0	0	2	2	2	0	5	0	1	1	0	0	0	1	0	0	3	1	1	0	0	3	0	0	0	0	0	0	22
Miguel	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Pool97	0	0	1	2	2	0	3	0	2	1	2	0	0	0	0	0	2	3	0	0	0	2	0	0	0	0	0	0	20
Salsi95	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	6
Salsi96	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	3	0	0	0	0	0	0	6
Salsi97	0	0	0	0	7	6	21	1	0	0	0	0	0	0	0	2	3	0	0	0	6	4	0	0	0	0	0	0	50
Malibu	0	0	0	0	0	1	0	0	0	0	0	0	0	7	3	0	5	0	0	2	0	0	0	0	1	6	1	0	26
Hilton	0	0	2	5	4	0	1	0	1	2	0	2	0	2	2	1	6	0	2	0	0	0	2	0	0	0	0	0	32
WhaleRock	0	0	1	11	21	2	2	3	9	1	0	0	9	0	0	0	0	0	0	2	0	0	0	0	0	2	0	3	66
NCAsthd	2	1	0	11	0	14	1	2	1	4	1	0	0	0	1	2	0	0	5	4	0	1	0	2	2	0	0	0	54
Total	2	1	19	43	49	25	45	10	18	16	3	3	9	11	6	8	30	7	11	8	8	15	4	2	3	8	1	3	368

APPENDIX I (cont.)

Locus = Omy325

	87	99	101	103	105	107	109	111	113	115	117	119	121	123	125	127	131	135	137	139	143	145	Total
Alisal95	0	0	0	0	1	0	0	3	8	2	0	18	0	0	0	1	0	0	0	0	0	1	34
Hilton94	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	2
Hilton95	0	0	3	0	0	8	1	2	12	4	0	3	1	2	0	12	0	0	0	0	0	0	48
Hilton97	0	0	0	0	0	3	0	0	1	5	0	0	1	5	0	7	0	0	0	0	0	0	22
Miguel	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	2
Pool97	0	0	1	0	0	1	0	0	1	3	0	0	0	5	1	7	0	0	0	0	1	0	20
Salsi95	0	0	0	0	0	2	0	0	2	0	0	0	0	0	0	2	0	0	0	0	0	0	6
Salsi96	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	4	0	0	0	0	0	0	6
Salsi97	0	0	0	0	11	0	0	0	0	4	5	5	0	0	0	15	0	1	5	4	0	0	50
Malibu	0	0	0	1	2	0	0	0	10	1	1	2	0	0	0	2	1	0	0	0	0	0	20
Hilton	0	0	2	1	1	0	1	0	10	4	0	0	1	1	0	2	1	0	0	0	0	0	24
WhaleRock	0	0	0	1	11	0	0	0	6	8	0	8	0	0	0	4	0	0	0	0	0	0	38
NCAstbd	1	1	3	7	8	2	0	11	3	1	4	4	1	2	4	2	0	0	0	0	0	0	51
Total	1	1	9	10	34	16	2	16	54	35	11	41	4	15	5	58	2	1	5	4	1	1	326

Locus = One#2

	194	204	206	306	208	308	210	212	312	224	226	230	232	234	236	238	240	242	244	246	248	250	252	254	256	258	260	262	264	266	268	270	274	282	Tot.
Alisal95	0	0	0	0	0	0	0	0	0	0	0	2	6	0	0	3	0	3	7	1	2	0	0	3	0	0	2	0	0	4	1	0	0	0	34
Hilton94	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Hilton95	1	0	3	1	1	1	9	7	3	0	2	0	1	0	2	4	0	1	3	0	0	0	0	2	2	0	0	0	0	0	1	3	0	1	48
Hilton97	0	0	2	0	2	0	2	0	0	0	0	0	2	0	2	7	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	22
Miguel	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	2
Pool97	0	0	1	0	0	0	3	1	0	0	0	0	3	0	0	5	0	0	0	0	0	0	2	0	1	0	0	0	0	2	1	1	0	0	20
Salsi95	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	6
Salsi96	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	6
Salsi97	0	0	0	0	0	0	0	0	0	0	0	16	2	0	0	0	0	3	11	1	0	0	0	9	1	0	5	0	2	0	0	0	0	0	50
Malibu	0	0	0	0	0	0	0	0	0	1	0	0	1	0	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8
Hilton	0	0	5	0	1	0	4	0	0	0	0	0	2	2	2	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22
WhaleRock	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	2	0	0	0	1	0	0	2	0	0	1	0	0	0	0	1	0	10
NCAstbd	0	1	1	0	0	0	0	0	0	2	0	0	1	1	3	2	4	5	4	2	5	3	3	1	4	1	1	0	1	0	1	2	0	0	48
Total	1	1	12	1	4	1	22	8	3	3	2	19	18	6	13	26	8	14	30	5	7	4	5	16	12	2	9	2	3	6	7	6	1	1	278

APPENDIX I (cont.)

Locus = Oneo8

	146	148	152	154	156	158	160	162	164	168	170	172	174	176	178	180	Total
Allsal95	0	0	3	0	0	21	0	0	0	0	10	0	0	0	0	0	34
Hilton94	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	2
Hilton95	0	0	2	0	0	35	5	0	0	1	2	2	0	1	0	0	48
Hilton97	0	0	0	0	2	14	6	0	0	0	0	0	0	0	0	0	22
Miguel	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	2
Pool97	0	0	0	0	0	14	4	0	0	0	1	1	0	0	0	0	20
Salsi95	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	6
Salsi96	0	0	0	0	0	5	1	0	0	0	0	0	0	0	0	0	6
Salsi97	0	0	0	0	0	26	24	0	0	0	0	0	0	0	0	0	50
Malibu	0	0	0	0	0	2	10	0	0	0	0	0	0	0	0	0	12
Hilton	0	0	0	0	0	1	18	2	0	0	1	0	0	0	0	0	22
WhaleRock	0	0	0	2	0	10	19	0	0	0	0	0	0	0	8	1	40
NCAstbd	4	1	0	0	0	16	15	10	2	0	0	1	1	0	0	0	50
Total	4	1	5	2	2	153	103	12	2	1	14	4	1	1	8	1	314

Locus = Oneo11

	141	143	145	147	149	153	Total
Allsal95	0	0	9	2	0	23	34
Hilton94	0	0	2	0	0	0	2
Hilton95	0	0	22	17	6	3	48
Hilton97	0	0	14	5	1	2	22
Miguel	0	0	1	1	0	0	2
Pool97	0	1	5	8	2	4	20
Salsi95	0	0	1	3	0	2	6
Salsi96	0	0	3	3	0	0	6
Salsi97	0	0	25	19	1	5	50
Malibu	1	0	8	5	0	0	14
Hilton	0	0	12	8	2	0	22
WhaleRock	0	0	32	2	6	0	40
NCAstbd	0	2	32	15	1	0	50
Total	1	3	166	89	19	39	316

APPENDIX I (cont.)

Locus = Oneµ14

	145	147	151	153	155	157	159	161	171	Total
Alisal95	0	11	1	1	10	0	0	11	0	34
Hilton94	0	0	2	0	0	0	0	0	0	2
Hilton95	0	1	8	6	16	12	0	5	0	48
Hilton97	0	2	0	11	0	6	3	0	0	22
Miguel	0	0	0	2	0	0	0	0	0	2
Pool97	0	2	3	4	4	6	0	1	0	20
Salsi95	0	0	0	0	4	2	0	0	0	6
Salsi96	0	0	0	0	2	4	0	0	0	6
Sals97	1	0	1	2	10	17	5	0	0	36
Malibu	0	0	8	2	0	4	0	0	0	14
Hilton	0	0	4	5	3	12	0	0	0	24
WhaleRock	0	0	2	0	24	8	0	0	0	34
NCAstbd	0	14	10	11	0	8	0	1	2	46
Total	1	30	39	44	73	79	8	18	2	294

Locus = Ots1

	233	237	239	241	243	151	155	159	161	163	165	167	169	171	175	183	185	Total
Alisal95	0	0	1	4	0	17	0	1	0	7	4	0	0	0	0	0	0	34
Hilton94	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	2
Hilton95	0	1	2	3	1	2	0	0	2	19	15	2	0	0	1	0	0	48
Hilton97	1	0	0	0	1	6	1	0	0	4	8	1	0	0	0	0	0	22
Miguel	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2
Pool97	2	0	0	1	0	6	1	0	1	4	5	0	0	0	0	0	0	20
Salsi95	0	0	0	1	0	0	0	0	0	3	2	0	0	0	0	0	0	6
Salsi96	0	0	0	0	0	3	0	0	0	1	2	0	0	0	0	0	0	6
Sals97	0	0	0	0	0	9	1	0	0	6	28	6	0	0	0	0	0	50
Malibu	0	0	0	1	0	0	0	1	0	6	7	0	0	0	0	0	1	16
Hilton	0	0	0	0	0	0	2	0	0	12	5	2	1	0	1	1	0	24
WhaleRock	0	0	0	0	0	0	0	0	0	12	13	0	0	1	0	0	0	26
NCAstbd	0	0	0	0	0	0	0	7	0	27	14	1	1	1	0	0	1	52
Total	3	1	3	10	2	44	5	9	3	102	105	12	2	2	2	1	2	308

APPENDIX I (cont.)

Locus = Ssa14

	126	128	130	134	136	140	142	144	146	148	150	152	158	166	Total
Alsal95	0	0	2	0	0	0	0	0	1	2	22	7	0	0	34
Hilton94	0	0	0	0	0	0	0	0	0	0	0	2	0	0	2
Hilton95	0	0	2	6	7	5	0	2	10	0	5	10	1	0	48
Hilton97	0	0	1	3	2	1	0	0	1	0	3	11	0	0	22
Miguel	0	0	0	0	0	0	0	0	0	0	1	1	0	0	2
Pool97	0	0	2	2	4	0	0	0	3	0	2	7	0	0	20
Salsi95	0	0	0	0	1	1	0	0	2	0	1	1	0	0	6
Salsi96	0	0	0	0	5	0	0	0	0	0	1	0	0	0	6
Salsi97	0	0	0	0	11	0	0	0	4	0	22	13	0	0	50
Malibu	2	1	0	0	0	0	0	0	1	0	5	1	0	0	10
Hilton	0	0	0	7	2	1	0	1	1	1	4	5	0	0	22
WhaleRock	0	0	7	1	0	0	0	0	0	0	7	1	0	0	16
NCAstbd	0	0	2	5	2	3	4	7	2	1	9	13	1	1	50
Total	2	1	16	24	34	11	4	10	25	4	82	72	2	1	288

Locus = Ssa289

	108	110	112	114	116	120	122	124	Total
Alsal95	0	21	10	0	0	2	1	0	34
Hilton94	0	1	0	0	0	0	1	0	2
Hilton95	1	13	11	5	0	11	6	1	48
Hilton97	0	2	7	6	0	1	5	1	22
Miguel	0	0	0	0	0	2	0	0	2
Pool97	0	2	5	7	0	3	3	0	20
Salsi95	0	2	0	2	0	1	1	0	6
Salsi96	0	5	0	0	0	1	0	0	6
Salsi97	0	44	4	0	0	2	0	0	50
Malibu	0	10	5	1	2	2	0	0	20
Hilton	0	11	5	1	0	4	2	1	24
WhaleRock	0	19	17	0	0	0	8	18	62
NCAstbd	0	4	23	4	6	3	6	8	54
Total	1	134	87	26	8	32	33	29	350

APPENDIX II - List of SYRTAC samples by basin and collection code with mtDNA haplotypes.

ation	Inventory #	Fish #	mtDNA
l 1995			
	82	A-05	3
	90	A-13	3
	81	A-04	3
	99	A-22	3
	88	A-11	3
	96	A-19	3
	87	A-10	3
	83	A-06	3
	85	A-08	3
	89	A-12	3
	94	A-17	3
	84	A-07	3
	80	A-03	3
	91	A-14	3
	30	A-02	3
	92	A-15	3
	93	A-16	3
n Cr. 1994			8
n Cr. 1995			
	2	H-02	1
	5	H-05	1
	12	H-12	1
	14	H-14	1
	17	H-17	3
	19	H-19	1
	15	H-15	14
	21	H-21	1
	23	H-23	8
	26	H-26	1
	37	H-29	1
	28	H-28	3
	45	H-37	3
	40	H-32	3
	47	H-39	3
	54	H-46	3
	56	H-48	3
	57	H-49	3
	62	H-54	3
	64	H-56	3
	68	H-60	3
	72	H-64	3
	78	H-68	1
	N/A	H-?	3

Population	Inventory #	Fish #	mtDNA
Hilton Cr. 1997			
	N/A	H-01	8
	N/A	H-02	3
	N/A	H-03	1
	N/A	H-04	1
	N/A	H-05	12
	N/A	H-06	12
	N/A	H-07	8
	N/A	H-08	3
	N/A	H-09	8
	N/A	H-10	8
	N/A	H-11	8
Long Pool/ Spill Basin 1997			
	N/A	4104E4913	3
	N/A	4109495D8	12
	N/A	410965D057	8
	N/A	4109DDC2E	3
	N/A	41096D617D	8
	N/A	4109512565	3
	N/A	41095A569C	12
	N/A	41095SDA5C	3
	N/A	41090C2146	1
	N/A	41095F5901	3
Salsipuedes 1995			
	31	S-01	3
	32	S-02	3
	79	S-04	1
Salsipuedes 1996			
	N/A	SU-01	8
	N/A	SU-02	8
	N/A	SD-02	5
Salsipuedes 1997			
	N/A	SD-02	5
	N/A	SU-02	8
	N/A	SD-03	8
	N/A	SU-04	5
	N/A	SD-04	8
	N/A	SU-05	8
	N/A	SU-09	8
	N/A	SD-05	8
	N/A	SU-11	8
	N/A	SU-26	8
	N/A	SU-15	3
	N/A	SU-16	3
	N/A	SU-18	8

Population	Inventory #	Fish #	mtDNA
Salsipuedes 1997 (cont.)			
	N/A	SD-06	8
	N/A	SD-08	5
	N/A	SD-09	8
	N/A	SD-10	3
	N/A	SU-22	8
	N/A	SU-21	8
	N/A	SU-25	8
	N/A	SU-29	8
	N/A	SU-33	3
	N/A	SU-01	8
	N/A	SU-10	8
	N/A	SU-31	8
San Miguelito 1997			
	N/A	SMD-01	3

APPENDIX III - List of previously published or reported mtDNA haplotypes found in streams of the Santa Ynez basin.

				mtDNA haplotypes										
Study	Location	Year	Age	1	3	5	6	8	9	10	12	13	14	total
Nielsen et al. 1994b	Alder Creek	1993	juv	2	1	6		6	4			6		25
	Franklin Cr.	1993	juv	2		4	5							11
	Fox Creek	1993	juv			2		12			4	2		20
CDFG (unpub. data)	Hilton Creek	1993	adult					1						1
	Hilton Creek	1993	1+	1	1					1			1	4
	Hilton Creek	1995	1+	5	3			1					2	11
JLN (unpublished data)	Peachtree Cr.	1993	YOY		2									2
ENTRIX (unpub. data)	Jameson Res.	1993	1+	4	7	13		1						25
ENTRIX, Inc. Fish Tech. Report for EIS/EIR Cachuma Project	Hilton Creek	1993	adult	1	2			1		1			1	6
	L. Cachuma	1993	na	3	3	1		2						9
	Santa Ynez R.	'93-'94	adult	1		2								3
	Salsipueses & El Jaro Crs.	1994	juv			12		11						23
USDA FS (unpub. data)	Indian Creek	1996	1+			1								1
	Devil's Creek	1995	1+		1			2						3
Total count				19	20	41	5	37	4	2	4	8	4	144